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A NEW CONCEPT IN MINIMALLY INVASIVE EMBRYO TRANSFER*

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Abstract

Considerable variation in embryo transfer (ET) catheter types, diverging opinions on their quality and functionality, complications following the insertion of catheters, low efficiency of the application of ET methods in humans, and their widely varying efficiency in animals demonstrate the need to improve ET methods and to look for new types of catheters. Such an opportunity is offered by the introduction of catheters made of new-generation biomaterials. This study was aimed to introduce a new generation of biomaterials into reproductive biotechnology. New-generation materials were compared with materials that have been used for many years, and the functionality of newly produced catheters was compared *in vivo*. Five types of biomaterials were tested: polycaprolactone (PCL), dibutyryl chitin (DBC), polypropylene (PP), polyethylene (PE) and polylactide (PLA). The study was carried out in two stages. Firstly, the basic utility parameters such as geometric stability, surface structure and catheter resistance were evaluated. Subsequently, the biocompatibility of selected biomaterials in embryo cultures was examined, and the development potential of the obtained blastocysts was evaluated. In the second stage, *in vivo* with live animals, the biomaterials were tested for biocompatibility and the obtained catheters were examined for their ET functionality. Efficiency with the use of the newly produced catheters was determined, the quality of the blastocysts obtained after embryo culture in the uterus was assessed, and oviducts were subjected to histopathological examination after embryo transfer. Of the tested biomaterials, only polyethylene (PE) showed adequate biological and material properties and proved suitable for production of ET catheters.

Key words: biomaterials, embryo, transfer, catheter, laparoscopy

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There has been an ongoing debate for many years regarding the effectiveness of embryo transfer methods in human and animal medicine. Embryo transfer (ET) is the final and one of the most critical stages of the *in vitro* fertilization (IVF) procedure (Sallam, 2005; Kuschnir et al., 2017; Niederberger et al. 2018). The type and quality of embryo transfer catheters is a very important factor. Many studies conducted on humans have compared embryo transfer catheters, but no conclusive answers have been offered, since various types of catheters have their advantages and disadvantages (Omidi et al., 2015; Lee et al., 2016; Bourdon et al., 2018; Cozzolino et al., 2018). According to the guidelines and rules for ET in humans, ET catheters should ideally be biocompatible, non-toxic to embryos, soft enough to prevent injury to the mucous membrane of the oviduct, uterus and cervix, but elastic enough to extend for the desired length into the lumen of the uterus or oviduct along the natural route; they should be as thin as possible and ensure that embryos can be transferred in a very short time (up to 2–4 minutes) and in a minimal amount of culture medium (around 20 µl) (Yao et al., 2009; Mains and van Voorhis, 2010; Spitzer et al., 2012; ESHRE, 2015; ASRM, 2017). Two types of catheters are used in humans: soft and rigid. Rigid catheters facilitate placement of the embryos into the uterine lumen, especially when the transfer is difficult, e.g. in the case of anatomical anomalies. However, the use of rigid catheters runs the risk of damage to the endometrium or oviduct, bleeding, causing the uterus or oviduct to contract, hindering implantation (Mains and van Voorhis, 2010; ASRM, 2017). Soft catheters are less convenient and more difficult to insert, but result in fewer complications in the form of mechanical damage to the endometrium and oviduct, and less bleeding (Ruhlmann et al., 2015; Schoolcraft, 2016; Conto et al., 2017). While human embryo transfer is performed with the catheters of known characteristics and exactly defined properties, there have been no studies conducted on animals. It should be stressed that a review of the literature indicated that there is wide variety of ET catheters used in animals. Various types of catheters are used in animals, e.g. human catheters or adapted and modified artificial insemination catheters; in small animals, intravenous Venflon catheters or Foley catheters are used (Galvin et al., 1994; Wallenhorst et al., 1999; Hazeleger and Kemp, 2001; Rátky et al., 2001; Angel et al. 2014; Martinez et al., 2015; Fonseca et al., 2016; Luo et al., 2019; Zheng et al., 2016). Attention is also drawn to low ET efficiency in humans, which ranges between 12% and 38% (Cozzolino et al., 2015; Niederberger et al., 2018). In animals, transfer efficiency varies widely from less than 20% to over 80% (Hazeleger and Kemp, 2001; Wallenhorst et al., 1999; Rátky et al., 2001; Martinez et al., 2015; Fonseca et al., 2016, Zheng et al., 2016; Peltoniemi et al., 2019). Considerable variation in catheter types, diverging opinions on their quality and functionality, complications following the insertion of catheters, and widely varying efficiency in animals demonstrate the need to improve ET methods and to look for new types of catheters. Such an opportunity is offered by the introduction of catheters made of new-generation biomaterials. For several years, many biological, synthetic and hybrid polymers have been used in the treatment of humans and animals (Maitz, 2015; Blazewicz and Marciniak, 2016). Despite such an extensive application of biomaterials, they have so far not been taken into account in reproductive biotechnology. The introduction of novel materials requires complex

research. It is necessary to verify toxicity to embryos, to perform material tests on the produced catheters, and to confirm their functionality in *in vivo* tests with animals. Our study used new-generation materials (PCL, PLA, DBC) and materials that have been known for many years (PE, PP). Many reports confirm the high biocompatibility of these materials towards somatic cells and all of them are routinely used in clinical (Aranaz et al., 2009; Orłowska et al., 2015; Maddah et al., 2016; Malikmammadov et al., 2018) and experimental medicine (Stodolak et al., 2010; Jang et al., 2012; Leszczynski et al., 2015). Accordingly, these materials were assumed to be biocompatible towards embryos, but it was necessary to directly verify this. A short contact time between the embryos and the biomaterial (15 min) is involved in the embryo transfer technique. Because embryos are sensitive to changing conditions, embryo transfer (ET) procedures strive to place them in their natural environment as soon as possible. The ET procedure takes up to 120 seconds in humans and up to 3–5 minutes in animals (Rátky et al., 1998; Wieczorek et al., 2015). Particularly harmful are rapid environmental changes, mainly temperature changes and hypoxia, which reduce the developmental capacity of embryos or cause them to die (Schoolcraft, 2016; Tıras et al., 2014), whereas prolonged transplantations have lower effectiveness, between 19% and 31% (Levi Setti et al., 2003; Schoolcraft, 2016). On the other hand, a rapid procedure reduces contact time between the transferred embryos and the catheter. It was therefore assumed that for a reliable determination of the toxicity of selected materials, the embryos would be placed in contact with the biomaterial for 15 minutes, which is longer than the potential contact between embryos and the catheter during an ET procedure. This study was aimed to introduce a new generation of biomaterials into reproductive biotechnology and to add to the progress of the area of embryo transfer. Although the dynamic progress of many areas of reproduction biotechnology can be observed, this progress has not affected embryo transfer, and the methods which were developed years ago are still in use (Niederberger et al., 2018; Peltoniemi et al., 2019). For this reason, it is necessary to employ new technologies and establish new and better procedures (Niederberger et al., 2018; Peltoniemi et al., 2019). The materials are characterized by high biocompatibility, desirable physical characteristics (elasticity and strength, proper surface structure), and the ability to be moulded into catheters with fixed dimensions. New-generation materials were compared with materials that have been used for many years, and the functionality of newly produced catheters was compared *in vivo*. The aim of this study was also to develop low-invasive methods of embryo transfer with the use of catheters made of new generation biomaterials.

Material and methods

Experimental design

All procedures were performed with the prior approval of II Animal Ethics Committee in Kraków, approval number 791/2010 and according to Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

The study was carried out in two stages (Figure 1). The first stage was conducted in two parts. The first part tested the physical and mechanical properties of biomaterials and the second part tested the biocompatibility of the selected biomaterials in *in vitro* embryo culture. Both parts of first stage were done simultaneously. The selected materials were tested for the possibility of producing catheters with the required properties and for their biocompatibility. In the second stage, the obtained catheters were examined for functionality in an *in vivo* model. The research model was established in pigs. Clinically healthy gilts were chosen as donors and recipients of embryos. They were 4 to 5 months of age, in the second or third cycle, before the first mating or AI. Donors were randomly selected from the same herd (from 250 potential donors with the same characteristics). The recipients and donors of embryos were subjected to routine synchronisation of estrus as it was described earlier (Wieczorek et al., 2015).

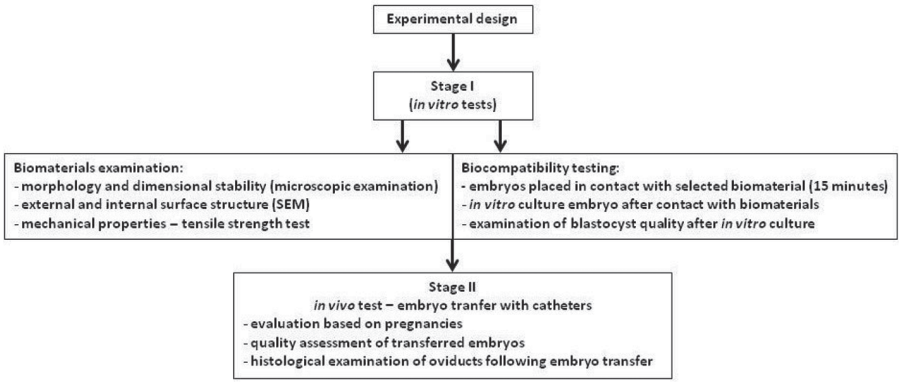

Figure 1. Examination protocol of *in vitro* and *in vivo* stages (experimental design)

Five types of biomaterials were tested: polycaprolactone (PCL), dibutyryl chitin (DBC), polypropylene (PP), polyethylene (PE) and polylactide (PLA). All of the polymeric materials used in the experimental process of manufacturing catheters were commercial; polycaprolactone (PCL, Sigma-Aldrich, Germany), polypropylene (Irganox® 1035, BASF, Germany), polyethylene (LDPE, BASF, Germany), polylactide (Ingeo® 3041D, NatureWorks, USA), dibutyryl chitin (Technical University of Łódź, Poland).

Stage I – *In vitro* evaluation

Stage I – Part I – physical and mechanical properties of biomaterials (testing the materials for the possibility of producing catheters)

To verify the possibility of producing catheters, the material characteristics of the tubes made from the dedicated materials were tested for their morphology and dimensional stability (microscopic examination), external and internal appearance (scanning electron microscopy), and mechanical properties (mechanical test). The

tests were done with the use of catheters (tubes) with a length of 40 mm, which were made of polyethylene with an external diameter of 1.0 mm, polypropylene with an external diameter of 1.76 mm, and polylactide (PLA) with an external diameter of 2.9 mm. Six replications were made for each tube material. Due to the lack of standards for catheters used for embryo transfer, standards for medical devices such as urological and intravenous catheters were applied. The specifics of the anatomical structure of the genitourinary tract were taken into account. The tests were performed on the basis of ISO standards applicable to medical materials and devices (ISO 10555-1, 2013; ISO 20696, 2018). The norms take into consideration the length and geometry of the catheter but also its mechanical properties (resistance to extension). The internal and external constancy of the catheter diameter seemed to be a legitimate object of study due to the need to maintain the stability of the shape during the operation of the catheter (keeping the catheter light when the embryo is transferred), especially considering that this medical instrument was obtained in a non-standard process of formation (Patent 225658).

Microscopic examination: observations under a stereomicroscope (100X) – morphology and dimensional stability

The aim of this examination was to determine the exact dimensions, and to test the dimensional stability and transparency of the catheter lumen. The observations (n=6 for each biomaterial) were made with a stereomicroscope (X100) (Alpha Vision) using FoxView software (Germany). The internal and external diameters of the examined tubes and the changes in these diameters were determined.

Microscopic examination – scanning electron microscopy (SEM) – external and internal structure

The surface structure was examined under a scanning electron microscope to visualise the external and internal morphology of the obtained tube. The surface morphology of the PE and PP tubes, which are in direct contact with pig tissues (oviduct) and embryos, was accurately determined. Tube samples were prepared by cutting along the longer axis of the catheter and affixing it with exposition of the interior. All the materials were sputter coated with carbon and observed under a microscope (FEI Nova NanoSEM 200, FEI EUROPE COMPANY, The Netherlands).

Mechanical tests – tensile strength test

Tensile strength tests were performed with a Zwick 1435 universal test machine (Germany), using a tensile test of 40 mm long tubes at an extension rate of 40 mm/min. Young's modulus (E) and tensile strength (R_m) were determined. The results are the average of 6 measurements of each tested material with standard deviation. The method of measurement of tensile strength was determined by the Polish norm ISO "Plastics. Determination of mechanical properties" (PN-EN ISO 527-3:2019-01). On the basis of the results of the first stage of the study, the thinnest catheters possible (PP 1.76 mm, and PE 1.0 mm) were prepared. It was impossible to manufacture a polylactide tube of the required parameters. The polypropylene (PP) catheter minimum diameter was limited by catheter manufacturing constraints (Patent

225658). The thinnest PP catheter possible to manufacture with the desired length of 20 cm had a diameter of 1.76 mm, while for PE catheters there were no material restrictions, so it was possible to obtain catheters of any diameter, even smaller than 0.5 mm (unpublished data). Initially, the material tests used a PP catheter of 1.76 mm in diameter.

Stage I – Part II – Biocompatibility testing of selected biomaterials based on embryo culture results and quality of the obtained blastocysts using TUNEL assays

In this part of the first stage of this experiment, embryos were cultured on the selected biomaterials and the quality of blastocysts obtained after embryo culture was assessed. Five pigs were donors of embryos. In five synchronised and superovulated donors, a total of 137 ovulations were observed on the oviducts and 123 embryos at the 2-4 blastomere stage were harvested. Following examination under a stereomicroscope, 117 embryos were cultured *in vitro* and 63 blastocysts were obtained. All the blastocysts were examined by TUNEL assay.

Culture and macroscopic examination of embryos

Embryos were surgically recovered according to a previously described protocol (Wieczorek et al., 2015). All obtained embryos were initially placed in one Petri dish. After the assessment, they were randomly assigned to experimental and control cultures (Groups PCL, DBC, PP, PE, PLA, and control).

The experimental groups and the control group were used for biocompatibility testing. In the experimental groups, 30 mm-diameter rings were prepared from the selected biomaterials. Embryo culture dishes were of equal diameter and the bottoms were lined with the prepared rings. Embryos obtained in the embryo culture medium were randomly placed in a Petri dish on a biomaterial medium. Embryos were placed in contact with the selected biomaterial for 15 minutes and transferred to a culture without biomaterial. The control group for these cultures were embryos cultured concurrently with the experimental groups without biomaterial contact (in the same culture dishes, not lined with biomaterial). Control embryos were also transferred to another dish to maintain identical conditions as in experimental groups. Cultures were incubated for 5 days in freshly prepared NCSU-23 medium (Petters and Wells, 1993) under a 5% CO_2 in an air atmosphere. During culture, the embryos were assessed daily under a stereomicroscope (at a magnification of over 100x) (Nikon SMZ 10A, Japan) in a laminar flow cabinet at around 30°C. The survival of the embryos was assessed based on their macroscopic examination and rate of development to the blastocyst stage. The quality of all blastocysts was examined using TUNEL assay, the number of cells in blastocysts was determined, and the apoptotic index was calculated.

Validation of the selected biomaterials for biocompatibility based on the quality of blastocysts examined using TUNEL assay

The analysis was performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). The number of stained cells (nuclei) in blastocysts was counted under an epifluorescence microscope (Eclipse E600, Nikon, Ja-

pan) using a 358–461 nm filter – the number of apoptotic nuclei was calculated with a 520 nm filter. The apoptotic index was calculated as the ratio of apoptotic nuclei to all nuclei.

Stage II – testing the selected biomaterials for biocompatibility and the catheters made from them for functionality *in vivo*

Biocompatibility evaluation in the *in vivo* test determined embryo transfer efficiency with a non-invasive laparoscopic method using prototype catheters made from the selected biomaterials, as well as analysing histological changes in oviducts 5 days after embryo transfer, and assessing the developmental potential of transferred embryos using TUNEL assay after 5 days of *in vivo* culture.

At this stage, the embryo donors and recipients were 80 gilts (56 embryo donors and 24 embryo recipients). The donors were additionally superovulated according to a previously described scheme (Wieczorek et al., 2015). Twenty-four hours before the procedure, feed but not water was withdrawn. Immediately before the procedure, a 20GA cannula was introduced into the marginal vein of the ear (Venflon BD 1.0 KD Medical, Poland). Embryos were surgically recovered according to a previously described scheme (Wieczorek et al., 2015).

***In vivo* test – evaluation based on pregnancies**

In this group of donors (n=48), a total of 650 ovulations were found on the ovaries and 578 embryos were obtained (89%). After preliminary assessment, 544 embryos (94%) were qualified for transfer and all of them were laparoscopically transferred to 19 recipients. An average of 28 embryos per recipient were transferred. The following criteria were adopted in the macroscopic evaluation of embryos: in the early stage of development, 2–4 cells embryos with correct morphology, transparent cytoplasm and intact zona pellucida were classified for further study. In the later stage of embryo development, large round symmetrical blastocysts with thin zona pellucida and separated trophoblast were classified for study.

Embryos at the 2–4 blastomere stage were introduced into the oviduct by laparoscopy, using a modified version of a previously reported method for laparoscopic transfer of embryos into the uterus (Wieczorek et al., 2015). Embryos were transferred with two sets of catheters: PP with a diameter of 1.76 mm and PE with a diameter of 1.0 mm. After stabilising the oviduct with a guide pin, the oviduct was punctured and a catheter with embryos was inserted 3–5 cm into the oviduct. Embryos were placed at the beginning of the catheter (1–2 cm) in a minimum volume of fluid (10–20 µl). Embryos were injected and deposited into the lumen of the right or left oviduct. After removal of the trocars, single simple PGA sutures were placed in the skin (PGA 1, DKO117PG, Yavo, Poland). Pregnancies were diagnosed by ultrasonography between 28 and 31 days after treatment.

***In vivo* test – quality assessment of transferred embryos using TUNEL assay**

Five recipients were euthanized on Day 5 after the transfer. For the tests, the recipients were randomly selected (Experimental design). The minimum number of animals necessary to obtain reliable results was used. Animals were subjected to general anaesthesia (Wieczorek et al., 2015). After making the animals totally un-

conscious, they were put down with intravenous 50–75 mg/kg iv sodium pentobarbital (Morbital 100 ml, Biowet, Poland). Uteri with oviducts and ovaries were collected. After 5-day *in vivo* culture, the transferred embryos (blastocysts) were flushed from the uterus. The control group were embryos obtained from donors and cultured *in vitro* as previously described. The quality of expanding blastocysts, grown *in vivo* and in *vitro*, was evaluated. Blastocysts were subjected to TUNEL assay, as described previously.

***In vivo* test – histological examination of oviducts following embryo transfer**

Oviducts were harvested from five recipients on Day 5 after embryo transfer, as described above. Histopathological examination of the fallopian tubes was planned to be performed in 4 pigs after the embryos were transplanted with a PP catheter and in 4 pigs after the embryos were transplanted with a PE catheter. The study was initially performed in 5 pigs (PE n = 4 and PP n = 1). On the basis of preliminary observations from one histological examination of the PP catheter and clinical observations and the course of transplantation in the recipients from the PP group, the use of subsequent recipients in this group, also intended for histopathological examination, was abandoned.

One oviduct after treatment with a polypropylene catheter and four oviducts after embryo transfer with a polyethylene catheter were collected for examination. The control group were five oviducts, into which no embryos were transferred. The collected oviducts were fixed in 10% formalin and then cut into 2–3 mm transverse sections using a microtome knife. The sections were placed into labelled histology cassettes while observing the sequence along the oviduct, placed in an automated tissue processor (Shandon Excelsior ES, USA) and embedded in paraffin. Paraffin blocks were sectioned into 2- to 3-µm-thick sections, which were stained with haematoxylin-eosin in an automated slide stainer (Shandon Varistain Gemini, USA) and examined under a light microscope. Histological changes were graded on a 4-point scale: 0 – none, 1 – small, 2 – moderate, 3 – considerable.

Statistics

Statistical analysis was performed with Statistica 8 software (StatSoft Inc) using two-way ANOVA, with a significance level of $P<0.05$. Statistically significant differences between the groups were determined with Student's t-test, and the consistency of results between the groups was measured by standard deviation (mean±SD).

Results

Stage I – *in vitro* testing

Stage I, Part I – physical and mechanical properties of biomaterials (testing the materials for the possibility of catheter making)

Microscopic examination: observations under a stereomicroscope (100X)

The key element taken into consideration in the evaluation of the possibility of using a given catheter was examination if the manufactured tube maintains a con-

stant thickness of the walls and to what extent the shape of the catheter's lumen changes (taking into account its desired length, the clearance may have collapsed). During catheter production, regularity and symmetry of the cross-section remained intact (Table 1). No significant changes in shape and dimensional stability were observed. Differences emerged between the polyethylene, polypropylene and polylactide catheters in surface structure, which was confirmed when the surface structure was examined under a scanning electron microscope (SEM, 3.1.1.2). Unlike the other catheters, the polylactide catheter was considerably porous, which was evident in macroscopic examination. In the stereomicroscopic examination, wall thickness was estimated to be around 100 μm (PE catheter), 300 μm (PLA catheter), and 243 μm (PP catheter) based on the measured diameters. The total internal diameter of the catheters was 1.5 mm (PP), and 0.8 mm (PE). It is assumed that such a thickness of the catheter walls allows smaller catheter dimensions and at the same time ensures a larger inner diameter, while maintaining the dimensions and not decreasing the lumen of the catheter.

Table 1. The microscopic evaluation of catheters (Δ-change)

Parameter	Material (n=6)		
	PE (1.0 mm)	PP	PLA
External diameter (mm)	1.0±0.05 ab	1.7±0.19 c	2.9±0.12
Diameter Δ (%)	3.8±0.02 a	8.5±0.05 c	2.5±0.03
Surface area Δ (%)	7.4±0.04 a	16.3±0.11 c	5.0±0.06

Statistically significant differences: a – PEvsPP ($P<0.001$), b – PEvsPLA ($P<0.001$), c – PPvsPLA ($P<0.001$).

Evaluation of surface condition – scanning electron microscopy (SEM)

The roughness of the internal surfaces of the catheter may have a significant impact on the safety of embryo transfer. On the other hand, observation of the external surface allowed for confirmation of the safety of tissues during the introduction and removal of the catheter during the medical procedure. Analysis of the external surface of polyethylene tubes revealed a lack of ideal smoothness and the presence of structural discontinuities in the form of irregular, shallow depressions that did not weaken the polymer structure. Furthermore, small cracks in the thin surface layer were noted. Morphological assessment of the inner surface confirmed the relative smoothness and regularity of the tested surface layer of PE catheters (Figure 2/1A,1B). Imaging of the outer surface of the polypropylene catheter showed many discontinuities and defects. There was considerable structural heterogeneity with relatively smooth areas and considerably rough areas. Small cracks (flakes) in the surface layer were also observed. At the same time, no deep perforations or major morphological changes were noticed. Inner surface morphology was found to vary, as evidenced by an uneven surface with shallow depressions, small bubbles of air in the polymer, and visible layers of melted material (Figure 2/2A,2B).

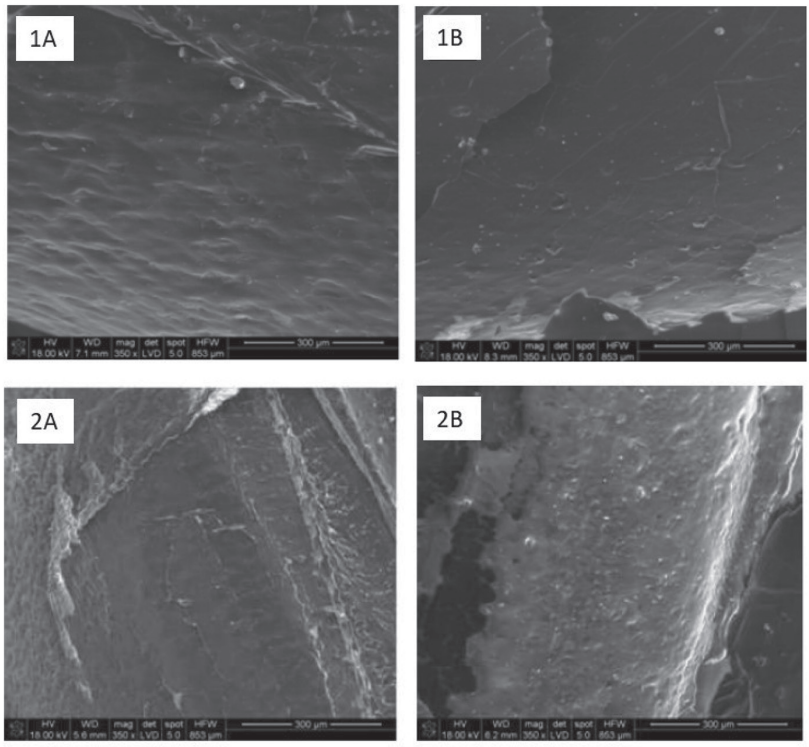

Figure 2. The external and internal surface of PE and PP catheters under scanning electron microscopy (SEM). Respectively: 1A – external surface of PE catheters, 1B – internal surface of PE catheters, 2A – external, surface of PP catheters, 2B – internal surface of PP catheters

Mechanical tests

In order to compare functional properties (surgical portability) of the obtained and tested catheters, the instruments were subjected to mechanical tests. In the strength test (Table 2), there were statistically significant differences between all the tested materials. Tensile strength was greatest for polyethylene tubes (PE 1.0 mm). It was greater than for PLA tubes ($P<0.05$), and seven times as high as for polypropylene catheters ($P<0.05$). Standard deviations showed that PE1.0 and PP catheters are highly homogeneous, whereas PLA tubes vary widely in strength. Thus, it has been indicated that the obtained catheters meet the requirements for safe insertion and removal of catheters for similar surgical instruments dedicated to urology or cardiology and intravascular catheters (ISO 10555-1:2013; ISO 20696:20180).

Young's modulus (Table 2) of elasticity showed that PE 1.0 catheters were the most elastic. Rigid catheters ensure the safety of the embryos in the lumen of the instrument, so it is necessary to verify this parameter. These catheters were of similar elasticity and no significant differences were found between them. PP catheters exhibited considerable elasticity, but this was significantly smaller compared to that of PE catheters (PP/PE1.0, $P<0.05$). PLA catheters were found to be rigid. The elas-

ticity of PLA catheters was several dozen times lower than that of PE catheters and around 18-fold lower than in PP catheters. Unfortunately, the mechanical properties (Rm, E) of catheters made of PLA are subject to a large standard deviation, which indicates low repeatability of material properties. This probably results from the forming method which was used.

Table 2. Evaluation of mechanical features of catheters: tensile strength and Young's modulus for PE 1.0 mm, PP1.76 mm and PLA catheters

Parameter	Material		
	PE	PP	PLA
Tensile strength (MPa)	66±3.4 ab	9±1.1 c	39±19.5
Young's modulus E (MPa)	96±18.8 de	117±10.3 c	2500±732

Statistically significant differences: a – PEvsPP (P<0.01), b – PEvsPLA ((p<0.01), c – PEvsPLA (P<0.05), d – PEvsPP (P<0.01), e – PEvsPLA (P<0.0005).

Stage I, Part II – Biocompatibility testing of selected biomaterials based on the results of embryo culture, and quality of obtained blastocysts using TUNEL assay

Culture and macroscopic examination of embryos

After 15 minutes of contact with polycaprolactone (PCL) and dibutyryl chitin (DBC), the development of all embryos was arrested and none developed into the morula and blastocyst stages. In the case of the other materials (PE, PP, PLA) and the control group, embryos developed to the morula and blastocyst stages. With PP, PLA, and the K-control group, a similar number of blastocysts was obtained, with no statistically significant differences between these groups (Table 3).

Table 3. Biocompatibility verification results of polycaprolactone (PCL), dibutyryl chitin (DBC), polypropylene (PP), polyethylene (PE), and K-control in embryo culture *in vitro* and determination of the degree of apoptosis of obtained blastocysts

Parameter	Material					
	PCL	DBC	PP	PLA	PE	K
Number of embryos in IVC	17	17	17	22	10	12
Number of 2–4 blastomere embryos	–	–	16	22	8	11
Number of morulae	–	–	11	17	8	10
% morula	–	–	65.00	77.3	80.0	90.0
Number of blastocysts	–	–	10	17	7	9
% blastocysts	–	–	58.6 a	77.3	70.0 b	83.3
Mean number of cell/blastocyst	–	–	30.6±6.5 a	31.4±2.4 cd	56.7±5.6 b	28.7±3.3
Apoptotic index (%)	–	–	3.3±0.03 a	4.3±0.04 c	13.3±0.02 b	2.3±0.03
Mean number of non-apoptotic cell/blastocyst	–	–	29.6±6.3 a	30.1±2.6 c	49.1±5.2 b	28.1±2.9

Statistically significant differences: a – PPvsPE, b – KvsPE, c – PLAvsPE, c – PLAvsK; P<0.05.

Validation of the selected biomaterials for biocompatibility based on the quality of blastocysts examined using TUNEL assay

The quality of PP, PLA and K blastocysts was similar as well, and no differences were found between these groups. In the case of PE, blastocysts had a greater number of cells per blastocyst (statistically significant differences PP/PE, statistically significant differences PE/K and statistically significant differences PE/PLA), with a considerably higher number of apoptotic cells per blastocyst (statistically significant differences PP/PE, statistically significant differences PE/K, and statistically significant differences PE/PLA) (Table 3).

Stage II

***In vivo* test – evaluation based on obtained pregnancies**

The results are presented in Table 4. Embryo donors (n=56) were divided into two groups. Embryos from the first group (n=48) were transferred to the recipients to obtain pregnancy, and the other eight donors produced embryos that were transferred to the recipients, from which after 5 days of incubation blastocysts were flushed from the uterus and from which oviducts for histological examination were also harvested.

Following embryo transfer with a PP catheter to four recipients, one pregnancy was obtained (25%). After the embryos were transferred with a PE catheter to 15 recipients, pregnancies were found in nine (9/15; 60%), but no statistically significant differences were noted between the groups.

Table 4. *In vivo* test of biocompatibility and functionality of catheters (number of ovulations, efficiency of embryo production, number of transmitted embryos, obtained pregnancies)

Parameter	Group		
	PP catheter	PE catheter	Total
Number of ovulations	138	512	650
Number of recovered embryos (%)	121 (87.7%)	457 (85.0%)	578 (89.0%)
Number of transplanted embryos (%)	121 (100.0%)	423 (93.4%)	544 (94.0%)
Number of recipients	4	15	19
Number of transplanted embryos/ one recipient	30.2±0.5	28.0±3.3	28.6±3.0
Number of pregnant recipients (%)	1 (25.0%)	9 (60.0%)	10 (53.0%)

No statistically significant differences between groups.

***In vivo* test – evaluation of transferred embryos using TUNEL assay**

The results are presented in Table 5 and in Figure 3. Embryos were collected from 8 donors, in which 100 ovulations were observed and 86 embryos at the 2–4 blastomere stage were obtained. After pr eliminary assessment, 80 embryos were transferred to five recipients, and the other six were retained for *in vitro* culture as a control group. Five days after the embryo transfer, recipients were euthanised, and blastocysts obtained during the 5-day *in vivo* culture were flushed from the uterus.

Out of 80 transferred embryos, 10 (12.5%) blastocysts were flushed. This method was also used to evaluate six blastocysts obtained *in vitro*. Embryos after incubation in the uterus and after *in vitro* culture had a similar quality, number of cells and apoptotic index. There were no statistically significant differences between the groups incubated *in vivo* and *in vitro*. An average of 56 cells were found in the flushed blastocysts that developed *in vivo*, 15% of which had apoptotic characteristics, and in the control group blastocysts had 56 cells on average, and the apoptotic index was 14.54%.

Table 5. Evaluation of the quality of embryos at the expanding blastocyst stage after development *in vivo* and *in vitro* on the basis of the total number of cell nuclei and the number of nuclei exhibiting apoptotic changes and the apoptotic index

Parameter		*In vitro* culture	*In vivo* culture
Number of blastocysts		6	10
Tunel	Total number of nuclei / one blastocyst	58.7±6.3	56.4±5.3
	Number of apoptotic nuclei / one blastocyst	8.8±2.6	8.2±1.9
	Apoptotic index (%)	15.0	14.5

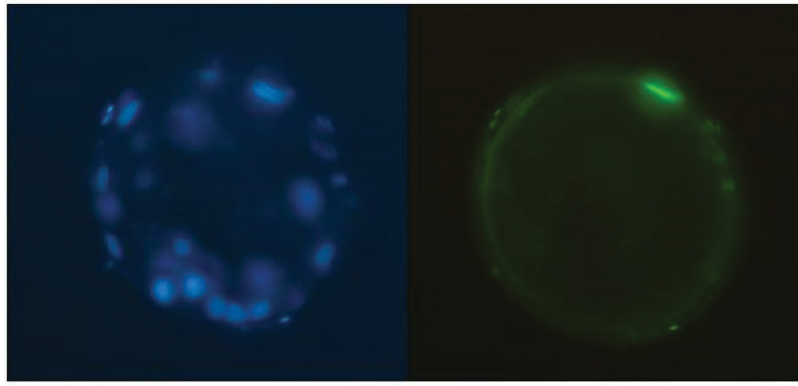

Figure 3. The blastocyst under TUNEL evaluation. The number of stained cells (nuclei) in blastocysts was counted under an epifluorescence microscope and the number of apoptotic nuclei was calculated with a 520 nm filter

***In vivo* test – histological examination of oviducts after embryo transfer**

The results are shown in Table 6 and Figure 4. Many histopathological lesions of different intensity were described in oviducts after transferring embryos with PP and PE catheters as well as in oviducts with no intervention. In the oviduct into which embryos were transferred using a PP catheter, the oviductal wall became damaged and extensive histopathological changes were found: changes in oviduct structure; the presence of epidermis and adipose tissue in the lumen of the oviduct; granulocytic infiltration in the wall; a cluster of granulation tissue in the wall, with visible loss of the oviductal wall after puncture. Such lesions were not observed after transferring embryos with PE catheters. (Table 6). Six pathological changes were described

in the oviducts into which embryos were transferred using a PE catheter. Similar lesions were also found in oviducts in which embryo transfer was not performed (control oviducts) (Table 6). Both in oviducts into which embryos were transferred and in control oviducts, small inflammatory infiltrations were observed. In terms of structural changes, the intensity of inflammatory infiltrations and patency of the oviductal lumen, the changes between the experimental and control groups were highly similar at 85–100%. In the case of oviducts after puncture and after introduction of the PE 1.0 catheter, additional small granulocyte infiltrations were found in the wall and oviductal lumen, as well as purulent lesions around the oviduct. For these lesions, no significant difference was found between the oviducts after puncture and control oviducts.

Table 6. Histological assessment of fallopian tubes after 5 days from embryo transfer

Histological evaluation	Experimental catheter PP (n=1)	Experimental catheter PE (n=4)	Control (n=3)
Normal structure	0.00	2.75±0.5	2.66±0.57
Inflammatory infiltrations	3.00	0.25±0.25	0.33±0.57
Preserved patency of fallopian tube	1.00	3.0±0.0	3.0±0.0
Granulocytic infiltration inside fallopian tube	3.0	0.25±0.57	0.0±0.0
Granulocytic infiltration in the wall of fallopian tube	3.0	0.5±0.57	0.0±0.0
Purulent lesions around fallopian tube	0.00	0.25±0.5	0.0±0.0
Epidermis and adipose tissue in the lumen of the oviduct	3.00	0.0±0.0	0.0±0.0
Loss of oviductal wall	3.00	0.0±0.0	0.0±0.0

Statistical analysis was not conducted for the oviduct after embryo transfer via PP catheter. No statistically significant differences between PE and control were found.

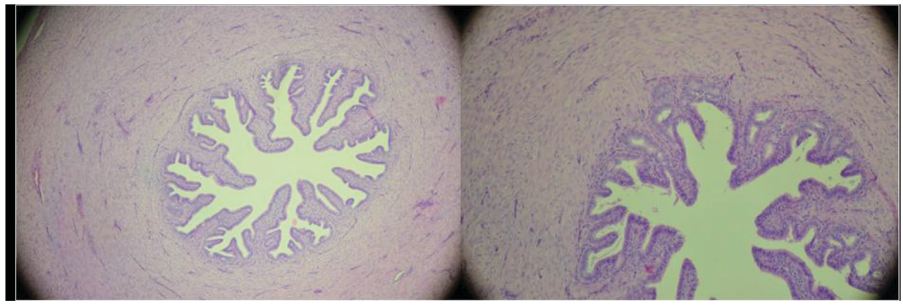

Figure 4. Histological examination of oviducts following embryo transfer – the clearance of oviducts preserved, mucosa in normal structure, visible granulocyte infiltrates in the mucosa (one of four oviducts after PE catheter transplantation)

Discussion

The toxicity tests yielded highly variable results. Polycaprolactone (PCL) and dibutyryl chitin (DBC) were found to be highly toxic to embryos and therefore the use of these biomaterials as a potential material for production of embryo transfer catheters was ruled out, as were their other applications in reproductive biotechnology. Polyethylene (PE), polypropylene (PP) and polylactide (PLA) have shown potential for use. However, the fact that the selected biomaterials are biocompatible with embryos during a short 15-minute contact does not preclude the negative effect of these biomaterials on the development of embryos. It is possible that a prolonged (e.g. several-day) contact of embryos cultured on these biomaterials may show that the biomaterials have low biocompatibility. This hypothesis will be subjected to further analysis. Biocompatibility of the materials was also confirmed by TUNEL assay of embryos.

The biocompatibility of the materials was also confirmed by the use of TUNEL assay on the embryos. TUNEL assay, used to identify inter-nucleosomal DNA strand breaks by enzymatic reaction using terminal deoxynucleotide transferase (TdT), catalysing the coupling of fluorescein isothiocyanate-labelled (FITC) nucleotides with free 3'-OH ends, allows apoptotic cells to be distinguished in a fluorescence microscope (Martinez et al., 2010; Samiec and Skrzyszowska, 2015). The phenomenon of apoptosis may appear already in earlier embryonic stages than the blastocyst as a response to abnormalities associated with embryological development (Betts and Madan, 2008; Haouzi and Hamamah, 2009). Based on the results obtained in the TUNEL assay, in PP, PLA and K there were no significant disturbances of cell division, and the obtained blastocysts showed a small and similar number of apoptotic nuclei and a small apoptotic index. In these groups, a very similar number of cells in blastocysts were also observed. Additionally in these groups, results comparable to other authors were obtained (Kidson et al., 2004; Gupta et al., 2007; Lin et al., 2017; Chen et al., 2018). In the case of PE, however, a much higher number of cells in blastocysts were shown to have a much higher apoptotic index. The high apoptotic index of the blastocyst after contact with PE may distort the correct quality of the obtained blastocysts and indicate false/apparent damage to embryonic cells during cultivation. On balance, when considering the total number of cells in the blastocysts and the apoptotic index, a much larger number of non-apoptotic cells were obtained compared to the other groups (PP, PLA and K) and also compared to those obtained by other authors (Kidson et al., 2004; Gupta et al., 2007; Lin et al., 2017; Chen et al., 2018). These results indicate the greater developmental potential of embryos after PE contact and may suggest the inductive properties of PE. However, this hypothesis requires confirmation in further studies. Disorders in embryological development also cause changes in embryo morphology manifesting mainly in the fragmentation of the cytoplasm. This anomaly that occurs in early cell division affects both the further development of the embryo, as well as the effectiveness of fertilisation, the percentage of blastocysts obtained, the number of cell nuclei (Pomar et al., 2005). Such changes in cultured embryos were not observed in our study, which additionally confirmed the good quality of the obtained blastocysts.

The results of the studies presented above indicate that the presence of biomaterials such as polypropylene (PP) and polyethylene (PE) is not detrimental to the development, survival and quality of pig embryos. Blastocysts cultured in the presence of PP were characterised by a similar number of cell nuclei compared to blastocysts cultured under standard conditions, whereas blastocysts cultured in the presence of PE had almost twice as many cell nuclei compared to control embryos. Only blastocysts from the experimental group, following contact with PE, showed higher apoptosis levels than in the control group. The presence of a large number of apoptotic nuclei in the blastocyst may suggest inappropriate *in vitro* culture conditions or the effect of other factors such as the quality of fertilised oocytes and the quality of semen used to inseminate female donors (Kidson et al., 2004). The toxicity of PE biomaterial would be confirmed during preliminary incubation of embryos, as in the case of the biomaterials polycaprolactone (PCL) and dibutyryl chitin (DBC), if embryos failed to develop after exposure to these two biomaterials, as well as if the number of cell nuclei was considerably reduced, which would be also indicative of lower blastocyst quality. PP and PE and the catheters made thereof were used for further studies. Although PLA was found to be biocompatible, its use for catheter production was excluded because of significant material defects. This was due to problems in moulding a catheter with a diameter smaller than 1.5 mm, and the considerable rigidity and porosity of the surface. The use of catheters of inadequate material quality results in mechanical damage to the endometrium and induces additional uterine contractions (Pomar et al., 2005; Tıras et al., 2014). Rigid catheters facilitate placement of embryos into the uterine lumen, especially when transfers are difficult, for example in the case of anatomical anomalies. However, this poses the risk of damage to the endometrium or oviduct, bleeding, stimulation of the uterus or oviduct to contract, and hinders implantation (Mains and van Voorhis, 2010). Some reports indicate that these complications have no effect on transplantation efficiency, indeed they increase transfer efficiency (Barash et al., 2003; Simon and Laufer, 2012; Singh et al., 2015). In terms of materials, embryo transfer in humans and animals relies on the use of catheters with minimal thickness, high tear resistance and elasticity, such that a catheter passes along the reproductive tract without mechanical damage to the mucous membrane of the uterus or oviduct (Martins et al., 2017). The surface structure test (SEM) revealed considerable differences in the surface structure between PE and PP catheters. PE catheters had small material defects, lacked an ideally smooth structure with few cracks in the surface layer, and had a smooth inner layer, but these had no effect on catheter functionality, which was confirmed in the third stage of the study. In the case of PP catheters, the considerable porosity of the outer wall and damage to the tube structure, with a smooth inner structure, had a significant effect during the stage of the study using the *in vivo* model. Because of the structural changes in these catheters, they were found to show low functionality and to be unsuitable for embryo transfer. It was assumed that a smaller catheter diameter, even with minimal porosity, would allow the catheter to be inserted without damaging the mucous membrane of the oviduct. Considering the easy moulding of catheters and the qualitative differences between the materials in the next stage, the *in vivo* test involved two catheters from extremely different materials: a PP catheter of 1.76 mm diameter and a PE cath-

eter of 1.0 mm diameter. This treatment considered the possible lack of success or low embryo transfer efficiency due to the considerable thickness of the catheter and structural heterogeneity of the PP tube surface, which can impede insertion of the catheter into the oviduct. A high embryo transplantation efficiency would confirm the appropriateness of choosing PP as a catheter material and prove that production of ET catheters does not require high quality materials. However, this catheter was eliminated due to the low efficiency after transfer of embryos via PP catheter and the considerable trauma to the oviduct shown in the histological examination of the oviduct after this catheter was inserted. Subsequently, a dedicated catheter with a PE diameter of 1.0 mm was used. The small diameter, flexibility and elasticity of this material allows the catheter with embryos to be inserted into the oviduct immediately after wall puncture, with the best embryo deposition depth ranging from 3 to 5 cm. Embryos were deposited at half the length of the oviduct. The use of this combination ensured high efficiency, which exceeded that reported by other authors. In pigs, the efficiency of laparoscopic embryo transfer varies from 10 to 40% (Rátky et al., 1998, 2001; Martins et al., 2017, Maged et al., 2018). Only Martinez and Galvin obtained over 70% efficiency following non-invasive embryo transfer (Martinez et al., 2015). The appropriate quality and functionality of the PE catheter as well as the efficiency of the described laparoscopic procedure of embryo transplantation into the oviduct was ultimately confirmed by the histological examination of oviducts and TUNEL assay of flushed blastocysts after 5-day *in vivo* culture. The histopathological examination showed minimal trauma to the oviduct after catheter insertion, and these changes had no effect on the oviduct structure and functionality. The TUNEL assay showed that the embryos remained viable after transfer into the oviduct. Like in the first stage of the study, the obtained blastocysts had a high cell number, a small number of apoptotic nuclei, and a low apoptotic index. These results confirm that the dedicated material and the proposed laparoscopic transfer procedure have no harmful effect.

Conclusions

The introduction of new materials in reproductive biotechnology requires complex research involving material tests, verification of toxicity in both *in vitro* laboratory tests and biological tests with live animals. Polycaprolactone (PCL) and dibutyryl chitin (DBC) cannot be recommended for application in reproductive biotechnology due to their high toxicity to embryos, nor can polylactide (PLA) and polypropylene (PP) because they fail to comply with material requirements. In the research concerning the introduction of new materials into reproduction, it seems that the most important thing is to confirm their biocompatibility in embryo culture, because the available biomaterials with confirmed biocompatibility with somatic cells may be highly toxic to embryos. Out of the tested materials, only PE was not toxic and had appropriate material properties, which makes it a suitable material for production of catheters.

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